Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis

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Abstract The release of two mitochondrial proteins, cytochrome *c* and apoptosis-inducing factor (AIF), into the soluble cytoplasm of cells undergoing apoptosis is well established. Using spectrophotometric determination of enzyme activity, the accumulation of adenylate kinase (AK) activity in the cytosolic fraction of apoptotic cells has also been observed recently. However, three isozymes, AK1, AK2 and AK3, have been characterized in mammalian cells and shown to be localized in the cytosol, mitochondrial intermembrane space and mitochondrial matrix, respectively, and it is unknown which one of these isozymes accumulates in the cytosol during apoptosis. We now demonstrate that in apoptotic cells only AK2 was translocated into the cytosol concomitantly with cytochrome *c*. The amount of AK1 in cytosol, as well as the amount of matrix-associated AK3, remained unchanged during the apoptotic process. Thus, our data suggest that only intermembrane proteins are released from mitochondria during the early phase of the apoptotic process.

Key words: Apoptosis; Mitochondria; Adenylate kinase; Cytochrome *c*

1. Introduction

Apoptosis is a mode of cell death, which is characterized by distinct morphological changes, including cell shrinkage, plasma and nuclear membrane blebbing, organelle compaction, condensation of chromatin and production of membrane-enclosed particles containing intracellular material known as 'apoptotic bodies'. Recently it has become clear that the mitochondria play a critical role in the early phase of apoptosis (for review see [1]). Thus, it is now well established that at least two proteins, cytochrome *c* and apoptosis-inducing factor (AIF), are released from mitochondria of apoptotic cells. AIF is a ~50 kDa protein, which can induce apoptotic changes in isolated nuclei [2]. Cytochrome *c* is a component of the cytoplasmic 'apoptosome' complex, which binds to Apaf-1; in the presence of dATP this binding leads to the sequential activation of pro-caspase-9 and other downstream caspases [3]. The mechanism of cytochrome *c* release is still unknown, although three different hypotheses have been proposed: (a) cytochrome *c* is released via specific channels in the outer mitochondrial membrane, which are opened by certain members of the Bcl-2-family of proteins, such as Bid, Bad or Bax [4]; (b) the opening of mitochondrial permeability transition pores, which results in the reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$), is responsible for cytochrome *c* release [2]; (c) leakage of cytochrome *c* occurs as a result of mitochondrial swelling and rupture of the outer membrane independently of loss of $\Delta\Psi_m$ [5].

Recently, using spectrophotometric determination of enzyme activity, adenylate kinase (AK) has been reported to accumulate in the cytosolic fraction of apoptotic cells concomitantly with the release of cytochrome *c* [6]. Adenylate kinase is a ubiquitous enzyme that contributes to the regulation of the homeostasis of the cellular adenine and guanine nucleotide pools. Three isozymes, AK1, AK2 and AK3, have been characterized to date [7]. All three AKs are nuclear-encoded proteins and synthesized in the cytoplasm. AK1 remains located mainly in the cytosol of different tissues. Mature AK2 and AK3, however, are imported into mitochondria, where they are sorted to different submitochondrial locations; AK2 is present in the intermembrane space whereas AK3 is located exclusively in the mitochondrial matrix and uses GTP instead of ATP as a phosphate donor [7]. It is unknown which one of the adenylate kinase isoforms accumulates in the soluble cytoplasm during apoptosis. In this study we demonstrate that in apoptotic cells AK2 was translocated into the cytosol concomitantly with cytochrome *c*. The amount of AK1 in cytosol remained unaltered as did the amount of the matrix-associated AK3. Thus, our data suggest that only intermembrane space proteins are released from mitochondria during the early phase of the apoptotic process.

2. Materials and methods

2.1. Cell culture and induction of apoptosis

Jurkat cells (human leukemic T cell line) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 3–4 days. Apoptosis was induced in cells by treatment with etoposide (6 μg/ml).

2.2. Immunoblot analysis

Untreated cells and cells treated with etoposide were washed twice in ice-cold PBS, resuspended in S-100 buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA), supplemented with protease inhibitors (0.1 mM PMSF, 10 mg/ml leupeptin, 5 mg/ml pepstatin A, 2 mg/ml aprotinin, 25 mg/ml calpain I inhibitor, 1 mM DTT), and incubated on ice for 10 min. Cells were centrifuged at 10 000 × *g* for 15 min at 4°C. The supernatant was then subjected to centrifugation at 100 000 × *g* for 1 h. For analysis of release of mito-

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Abbreviations: AIF, apoptosis-inducing factor; AK, adenylate kinase

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chondrial proteins, 100 μl of post-mitochondrial cytoplasmic extract (10 000 × *g* supernatant) as well as mitochondrial pellets were mixed with 5 times concentrated Laemmli's loading buffer, boiled for 4 min and subjected to 12% SDS-PAGE at 130 V followed by electroblotting to nitrocellulose (0.2 μm) for 2 h at 100 V. Membranes were blocked overnight in a buffer (50 mM Tris, pH 7.5, 500 mM NaCl) containing 1% bovine serum albumin and 5% non-fat dried milk. Then they were sequentially probed with the anti-cytochrome *c* (1:2500), anti-AK1, AK2 and AK3 (all at 1:2000) antibodies, followed by 1 h incubation with secondary IgG (1:10 000), and then visualized by ECL according to manufacturer's instructions.

3. Results and discussion

Although the release of cytochrome *c* from mitochondria has been observed in different experimental models of apoptosis, the kinetics of this release are not identical in every system [8]. Therefore we first examined cytochrome *c* release in our experimental system. Mitochondrial and cytosolic fractions were isolated at different time points from Jurkat cells incubated with etoposide, and equal amounts of protein were subjected to SDS-PAGE. Immunoblot analysis revealed etoposide-induced release of cytochrome *c* from mitochondria and its accumulation in the cytosol within 1–2 h of incubation (Fig. 1). Accumulation of a considerable amount of mitochondrial cytochrome *c* in the cytosolic fraction was observed at 4–5 h of incubation (Fig. 1). At 6 h, only small amounts of cytochrome *c* remained in the mitochondrial fraction of etoposide-treated cells. Activation of caspase-3-like proteases, measured by cleavage of a fluorogenic substrate (DEVD-AMC), was initially detected at 3 h after treatment, whereas DNA fragmentation was observed at 5 h (data not shown). Thus, in etoposide-treated Jurkat cells, cytochrome *c* release preceded the activation of class II caspases (DEVD-ases).

To address the question of which AK isozyme is responsible for the increase in adenylate kinase activity in the cytosolic fraction of apoptotic cells [6], the same membranes were probed with antiserum against different adenylate kinases. As described above, AK1 is present mainly in soluble cytoplasm of different tissues, such as skeletal muscle, brain and erythrocytes. In agreement with these observations, a faint band of AK1 was found in the cytosol of Jurkat cells, whereas no AK1 protein was present in the mitochondrial fraction (Fig. 1). The amount of AK1 in the cytosol did not change markedly during 6 h of incubation with etoposide. These results suggested that the increased AK activity in the cytosol of apoptotic cells was not coupled to an increased amount of AK1.

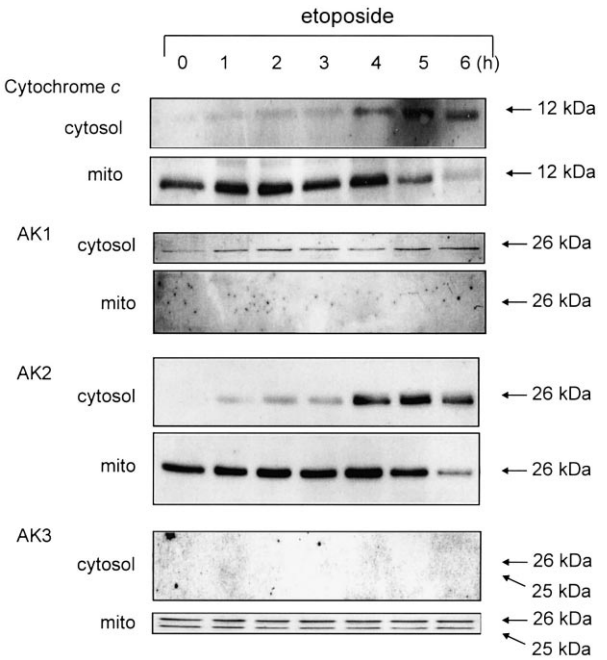

Fig. 1. Time-course of cytochrome *c* and adenylate kinase release in etoposide-treated cells. The 10 000 × *g* supernatants and pellets from etoposide-treated cells were prepared for Western blotting. The membranes were probed with antiserum against cytochrome *c*, adenylate kinase 1 (AK1), adenylate kinase 2 (AK2) or adenylate kinase 3 (AK3).

Another isoform of adenylate kinase, AK2, exists in the mitochondrial intermembrane space of many cell types. Small amounts of this protein can also be found in the cytosol of liver cells [7]. The low AK activity in the cytosol of different cell types [6] could thus be dependent not only on the presence of AK1, but also on the presence of trace amounts of AK2 [7]. However, using specific antiserum, AK2 was found only in the mitochondrial fraction of untreated Jurkat cells (Fig. 1). After treatment with etoposide, accumulation of AK2 in the cytosolic fraction occurred simultaneously with the accumulation of cytochrome *c* (Fig. 1). At 6 h of incubation only trace amounts of AK2 remained in the mitochondrial fraction.

As mentioned above, AK3 is localized mainly in the matrix of mitochondria. Western blot analysis using antiserum against AK3, revealed two bands with a molecular weight of 26 and 25 kDa, respectively, in the mitochondrial fraction of Jurkat cells (Fig. 1), which is in agreement with previously published results [7]. No AK3 was observed in the cytosolic fraction. Moreover, the amount of mitochondrial AK3 was stable during the time of investigation, suggesting that it was not released from the mitochondrial matrix.

Thus, the above experiments have shown that two proteins located in the mitochondrial intermembrane space, cytochrome *c* and AK2, are released concomitantly in etoposide-treated Jurkat cells. The release of both enzymes seems to be a general phenomenon in apoptosis, since it was also observed in Jurkat cells treated with agonistic anti-CD95 antibodies, staurosporine, or multimeric α-lactalbumin (data not shown). The mechanism for the release of cytochrome *c* and AK2, as well as AIF, from mitochondria in cells undergoing apoptosis is still unclear. As mentioned above, several possible pathways for the release of mitochondrial constituents have been suggested [1]. Recently it has been reported that this release occurs prior to mitochondrial ultracondensation, formation of outer membrane discontinuities and loss of electrical potential across the inner mitochondrial membrane [9], which would be in agreement with early work suggesting that mitochondria are structurally intact during the initiation stage of the apoptotic cascade [10]. The importance of rupture of the outer mitochondrial membrane and dissipation of $\Delta\Psi_m$ for the leakage of mitochondrial constituents is still unknown. Some blood cells can survive and maintain clonogenicity with reduced mitochondrial membrane potential [11]. It is also possible that the order of cytochrome *c* release, functional changes in mitochondrial membranes and commitment to apoptosis can vary between cell types and apoptotic stimuli [11].

As mentioned above, cytochrome *c* released into the soluble cytoplasm functions as a component of the 'apoptosome'

complex. Its ability to do so is based on stringent allosteric interactions between cytochrome *c* and other cytoplasmic factors, including dATP, and does not depend on redox reactions of cytochrome *c* [12]. The function of adenylate kinases in normal cells is restricted to the phosphorylation of AMP to ADP, which is further phosphorylated to ATP either by glycolytic enzymes or through oxidative phosphorylation. The functional role for AK during the apoptotic process is unknown. It can be speculated, however, that AK, once released from mitochondria, may affect the levels of ADP and ATP or dATP, in conjunction with ribonucleotide reductase, and thereby contribute to the apoptotic process via the 'apoptosome' complex. It could also be that a possible role for AK in the apoptotic process does not relate to the 'normal function' of AK in cells. Interestingly, a new adenylate kinase, AK4, was recently isolated from brain and shown to act specifically on energy metabolism rather than in the control of homeostasis of the ADP pool [13]. Investigation of the role of AKs in apoptosis is part of ongoing research in our laboratory.

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